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November 30, 1990

Summaries of Presentations at the
Workshop on the Elimination of Aflatoxin

The Agricultural Research Service and the Corn Growers Association sponsored a
Workshop on the Elimination of Aflatoxin at the Stouffers Concourse Hotel in
St. Louis on October 17-18, 1990. The Workshop included sessions on:

        Ecological Relationships and Agronomic Practices
        Effect of Insects and Their Control
        Biocompetitive Approach to Aflatoxin Elimination
        Delineation and Control of the Pathway of Toxin Formation
        Breeding for Resistance

The workshop was of immediate and direct concern to four commodity groups,
peanuts, corn, cottonseed, and nut trees. Representatives of these groups
have been influential in raising Congressional awareness of the aflatoxin
problem and the need to place adequate resources with ARS to support research
to prevent aflatoxin contamination in food and feed. It is recognized that
the major effort needs to be placed on preventing the problem in the field
rather than allowing contaminated commodities to enter the marketplace.

All presentations at the Aflatoxin Elimination Workshop were of studies
supported by the Agricultural Research Service, either within ARS laboratories
or as cooperative studies with scientists from universities.

A fourth workshop is planned for October 1991, in the Atlanta area. Both
suggestions and questions regarding this workshop should be addressed to:

        Jane F. Robens
        National Program Leader
        Food Safety and Health
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        Beltsville, Maryland 20705

AFLATOXIN ELIMINATION WORKSHOP
October 17-18, 1990
Stouffers Concourse Hotel
St. Louis, Missouri

Wednesday

Pre-meeting Coffee and Doughnuts

8:30 am **Welcome to St. Louis**
Robert Gebhards, Corn Growers Association
J. Robens, U.S. Department of Agriculture, Agricultural Research
Service (USDA/ARS)

8:45 am **Ecological Relationships and Agronomic Practices**

Population Dynamics of A. flavus and A. parasiticus in Soil,
D. Wicklow, USDA/ARS, Peoria, Illinois

Ecology of Aspergillus flavus in Iowa Crop Fields, J. F. Shearer,
L. E. Sweets, and L. H. Tiffany, Iowa State University, Ames

Ecological Relationships of Aspergillus flavus in Tree Nut
Orchards, T. Michailides, University of California, Davis

Effective Management Strategies for Prevention of Aflatoxin in
Peanuts, R. Cole, USDA/ARS, Dawson, Georgia

The Role of Insects in the Infection Process, B. D. Barry,
USDA/ARS, Columbia, Missouri

10:20 am **BREAK**

10:40 am Sap Beetles and Their Control, P. Dowd, USDA/ARS, Peoria, Illinois

Climate and Weather Factors as They Influence Aflatoxin
Contamination of Corn, N. W. Widstrom, USDA/ARS, Tifton, Georgia

11:30 am **Panel Discussion -**
What can ecological control/agronomic practices accomplish?
Chair: J. Robens
Panel: D. Wicklow, L. Sweets, T. Michailides, R. Cole, B. Barry,
P. Dowd, and N. Widstrom

12:30 **LUNCH AND POSTERS**

POSTERS

1. N. Zummo, ARS, Mississippi State, MS
2. A. Zaltzman, Sorting Technology, Inc., Pocatello, ID
3. M. Kang, Louisiana State Agricultural Experiment Station, Baton Rouge
4. J. Dorner, ARS, National Peanut Research Center, Dawson, GA
5. D. M. Wilson, University of Georgia, Tifton, GA
6. M. Klich, ARS, Southern Regional Research Center, New Orleans, LA
7. J. Mellon, ARS, Southern Regional Research Center, New Orleans, LA
8. J. Neucere, ARS, Southern Regional Research Center, New Orleans, LA
9. H. Zeringue, ARS, Southern Regional Research Center, New Orleans, LA

1:30 pm **Biocompetitive Approach to Aflatoxin Elimination**
         Chair: J. Richard, USDA/ARS, Peoria, Illinois

         Field Release of an Atoxigenic Strain of Aspergillus flavus,
         P. J. Cotty, USDA/ARS, New Orleans, Louisiana

         Biocompetitive Approach to Aflatoxin Prevention in Peanuts.
         R. Cole, J. W. Dorner and P. D. Blankenship, USDA/ARS, Dawson,
         Georgia

         Preliminary Evaluation of Paecilomyces sp. as Potential Biocontrol
         Agents, D. M. Wilson and M. E. Will, University of Georgia, Tifton

         Management of Aflatoxin Contamination of Cottonseeds Using
         Endophytic Bacteria, I. J. Misaghi, University of Arizona, Tucson

         Collection of Antagonists from Tree Nut Orchards and Their Use for
         Biological Control of Aspergillus flavus, N. F. Sommer, University
         of California

3:30 pm **BREAK**

4:00 pm **Panel Discussion**
         Safe and Acceptable Biocompetitive Approaches
         Chair: J. Richard
         Panel: P. Cotty, R. Cole, D. Wilson, I. Misaghi and N. Sommer

Thursday

Pre-meeting Coffee and Doughnuts

8:00 am   Delineation and Control of the Pathway of Toxin Formation
          Chair: J. Hunter, Procter and Gamble Company

          Elucidation of the Biosynthesis of Aflatoxins, D. Bhatnager,
          USDA/ARS, New Orleans, Louisiana

          Bioregulatory Control of Aflatoxin Synthesis, T. E. Cleveland,
          USDA/ARS, New Orleans, Louisiana

          Characterization of a Gene for Aflatoxin Biosynthesis, G. A.
          Payne, North Carolina State University, Raleigh

10:00 am   BREAK

10:20 am   Panel Discussion
           Aflatoxin Biosynthesis
              Chair: J. Hunter
              Panel: E. Cleveland, G. Payne, and D. Bhatnager

11:30 am   Breeding for Resistance
           Chair: C. Murphy, USDA/ARS

           Techniques for Identification of Resistance to Preharvest
           Aflatoxin Contamination in Peanut, C. C. Holbrook, USDA/ARS,
           Tifton, GA; D. M. Wilson, Tifton, GA; W. F. Anderson, Yuma, AZ;
           M. E. Matheron, Tifton, GA; M. E. Will and A. J. Norden,
           Gainesville, FL.

12:00   LUNCH

1:00 pm   Breeding for Resistance to Aspergillus flavus, G. Scott, USDA/ARS,
           Mississippi State, Mississippi

           A New Beginning in Examining Maize Genotypes for Resistance to
           A. flavus, D. Wicklow, USDA/ARS, Peoria, Illinois

           Distribution of Aflatoxin Contamination Among Single Kernels from
           Individual Plants and Among Single Plants, J. W. Dorner, R. J.
           Cole and P. D. Blandenship, USDA/ARS, Dawson, Georgia

           Screening Maize Germplasm for Higher Levels of Resistance to A.
           flavus and Aflatoxin Production, T. Rocheford, University of
           Illinois, Urbana

           Dry Seed Resistance in Peanuts, T. Stalker, North Carolina State
           University, Raleigh

3:00 pm   BREAK

3:20 pm   Panel Discussion
           Can We Identify Useful Resistance in Germplasm?
              Chair: C. Murphy, ARS
              Panel: C. Holbrook, T. Stalker, D. Wicklow, T. Rocheford,
                      G. Scott, and R. Cole

4:30 pm   Adjournment of Workshop

           Meeting of Technical Advisory Group

AFLATOXIN ELIMINATION WORKSHOP
Stouffers Concourse Hotel
St. Louis, Missouri
October 17-18, 1990

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10-31-90

Population dynamics of Aspergillus flavus and A. parasiticus in soil.

Donald T. Wicklow
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Sclerotia and conidia produced by A. flavus and A. parasiticus represent important sources of inoculum in the disease cycle of these aflatoxin-producing fungi. We have been investigating the survival of A. flavus sclerotia and conidia buried in sandy soils at the Illinois River Valley Sand Field (IRVSF), Kilbourne, IL and the Coastal Plains Research Station, Tifton, GA. This allows us to contrast survival in a temperate latitude vs. near-subtropical latitude on an equivalent soil type. Initial dilution platings (1 x 10 2) of soil samples collected from cultivated fields near Tifton revealed 100 - 7400 colony forming units (CFU's) of A. flavus per gram of soil while we failed to detect A. flavus in IRVSF soils. Is there a reduced rate of survival for A. flavus sclerotia and conidia in northern latitudes? If so, this could explain, in part, why aflatoxin contamination of midwest corn occurs less frequently than corn grown in southern Georgia? We wanted to determine the fate of sclerotia vs. conidia buried in soil for up to 3 years in the absence of a susceptible host crop (i.e. maize, peanuts) in order to apply this information in planning crop rotation sequences. We also wanted to learn if the sclerotia are attacked by any naturally occurring soil microbes that might be used in biocontrol. The sclerotia (@ 18/g soil) and conidia (@ 10,000/g soil) were blended into soil collected from each burial site. Individual stainless steel "tea balls" were filled with @ 60 grams of inoculated soil and buried in October 1986. In Georgia the numbers of CFU's derived from conidial populations dropped sharply between April and October 1987. In Illinois the conidial CFU's remained unchanged during this same interval but declined sharply the second year. At the conclusion of the experiment (October 1989) we were unable to detect A. flavus or A. parasiticus in 22 of 24 Georgia samples or in 15 of 21 Illinois samples. In soils inoculated with sclerotia, Aspergillus populations grew to 1,000 - 32,000 CFU's/g (Georgia) and 3,000 - 79,000 CFU's (Illinois), indicating that the sclerotia had germinated beneath the ground. By October 1989 Aspergillus populations in sclerotia-ammended soils had declined somewhat in Georgia (300 - 7,800 CFU's/g) but remained high in Illinois (1,900 - 83,600 CFU's/g). The data show that Aspergillus sclerotia and conidia survive burial in Illinois as well as in Georgia. There are a number of potential explanations as to why Aspergillus populations in cultivated sandy soils in Illinois do not match those in Georgia.

Paecilomyces lilacinus was identified as the principal fungal colonist of Aspergillus sclerotia buried at each location while Bacillus subtilis colonized up to 42% of the sclerotia that were buried for 36 months in Georgia. In initial tests of their potential as biocontrol agents, P. lilacinus colonized and rotted the sclerotia, preventing sclerotium germination. Bacillus subtilis, however, failed to prevent sclerotium germination. Aflavinine metabolites 'leak' from A. flavus sclerotia and also show mild antibiotic activity to B. subtilis.

Ecology of *Aspergillus flavus* in Iowa crop fields. Judy F. Shearer, Laura E. Sweets, Lois H. Tiffany and Noelle K. Baker.

In 1988 following an unusually hot, dry growing season, aflatoxin contaminated corn was a serious problem in Iowa. Samples of soil and of stalk and cob debris were collected from 40 aflatoxin contaminated corn fields in eight Iowa counties following the 1988 harvest. Additional samples were collected from these fields in the spring 1989, fall 1989, and spring 1990.

Soil samples were tested for the presence of *Aspergillus flavus* by sprinkling approximately 0.5 gm of soil onto the surface of M3S10B agar plates. Pieces of cob and stalk pith approximately 2.0 cm in diameter were pulled out of freshly broken cob and stalk samples and plated on M3S10B agar plates. Plates were incubated at 37 C for 3 days and visually examined for presence of *A. flavus* colonies. *A. flavus* was detected in soil samples from all fields collected at the end of the first three sampling times and from 85% of the fields in spring 1990.

Cultures of *A. flavus* isolated from each of the sampling periods were tested for ability to produce aflatoxin and for sclerotial production. Sixty five percent of the isolates from the fall 1988 collections were potential toxin producers, whereas approximately one half that number were detected in spring 1990 isolates. While only 23% of the isolates from fall 1988 were shown to be sclerotial producers, over 50% of the isolates from all subsequent collections have shown good sclerotial production. Although *A. flavus* isolates from Iowa have been shown to have the ability to produce sclerotia, there have been no field collections to date which have indicated that sclerotia develop in the standing crop as has been shown in southern United States.

About 70% of the cob pieces from both fall 1988 and spring 1989 samplings were positive for *A. flavus*. However, with the stalk pieces 42% of the fall samples and 84% of the spring 1989 samples were positive for *A. flavus*.

Detection and monitoring of *A. flavus* in crop soils from three Iowa State research farms was begun in the fall 1989. At each of the three locations, soils were sampled from randomized plots with different tillage practices and cropping regimes. While levels of *A. flavus* were different at the three geographically separated sites, neither tillage practices nor crop rotations appeared to significantly effect the incidence of *A. flavus* in the soils.

Competition studies using dual plate cultures of *A. flavus* and potential fungal competitors from agricultural soils in Iowa are under preliminary investigation. *A. flavus* grows and sporulates well at a wide range of temperatures. While a few soil fungal isolates show competitive ability against *A. flavus* at 25 and 30 C none of the isolates tested to date are able to outgrow and outcompete *A. flavus* at high temperatures and low water potentials typical of the stress conditions which lead to aflatoxin contamination of corn.

GENETIC VARIATION IN THE RESISTANCE OF VARIOUS CULTIVARS OF PISTACHIO, ALMOND, WALNUT, AND PECAN TO ASPERGILLUS FLAVUS/PARASITICUS AND DETERMINATION OF ENVIRONMENTAL AND ECOLOGICAL FACTORS NECESSARY FOR STABLE AND EFFICIENT PREHARVEST AFLATOXIN SUPPRESSION

Themis J. Michailides, Department of Plant Pathology, University of California, Berkeley/Kearney Agricultural Center, Parlier, CA 93648; **Noel F. Sommer**, Department of Pomology, Univ. of California, Davis, CA 95616; and **Petter J. Cotty**, USDA, ARS, Southern Regional Center, New Orleans, LA 70179.

The influence of cultural practices (type of irrigation and presence of cover crop) on the quantities of soil dust deposited on pistachio leaves and their effects on the microflora composition were studied. Eight commercial pistachio orchards irrigated by microjets, by flooding, or by sprinklers on pull hoses, each with and without a cover crop were included in these studies. Five replicated samples of fruits were collected bimonthly, washed in sterile water, and washings were plated on acidified PDA dishes. All the microorganisms recovered in the dishes were identified and counted. Propagules of Aspergillus niger, A. flavus, and other Aspergillus spp. were recovered in samples collected only from the beginning of August through mid September. Incidence of Aspergillus spp. was higher in orchards irrigated by flooding or sprinklers. Amounts of dust deposited on pistachio trees was 0.31 and 0.21 g/20 leaflets for orchards irrigated with microjets or flooding, respectively, and 0.13 g/20 leaflets (significantly smaller) for orchards irrigated by sprinklers. For all sampling dates, regardless of the irrigation type, orchards with cover crops usually had significantly smaller amounts of dust deposited on the trees than those in orchards where the soil was disced. Levels of Aspergillus flavus and other Aspergillus spp. in these orchards are presently being summarized. At commercial harvest, replicated samples of mature nuts were collected to determine aflatoxin levels as influenced by cultural practices. Results are not yet available.

In another study, antagonistic microflora were collected from a) soil of pistachio orchards, b) air samples of pistachio orchards, and c) pistachio fruit and leaf samples. An Anderson spore trap was used biweekly in 8 pistachio orchards, operating for 15 min in each orchard. Propagules of antagonists were recovered on 6 each PDA dishes inoculated with approximately 50 colonies of A. flavus and on 6 noninoculated dishes. Microorganisms exhibiting inhibition zones against A. flavus in PDA dishes were isolated and used in biocontrol experiments in an experimental pistachio orchard at Kearney Agricultural Center. Replicated samples of pistachio fruit were frozen for aflatoxin analysis.

Thyanta stinkbugs (Thyanta pallidovirens), which are very common in pistachio orchards, were used in transmitting A. flavus. The insects were fed on a sporulating 10-day-PDA culture of A. flavus for 24 hrs and then caged with healthy pistachio clusters. The pistachio fruits were collected 25 days later and were frozen for aflatoxin analysis. Insects fed on a 10-day-PDA dish without A. flavus and caged with pistachio clusters served as controls.

Insects Related to Preharvest Maize
Contamination with Aflatoxin

Dean Barry and L. L. Darrah

The spores of *Aspergillus* spp. which produce aflatoxin are omnipresent and allow insects associated with maize (*Zea mays* L.), especially those insects inhabiting the ear, an opportunity to vector the fungi. Besides the vectoring, insect feeding enhances the environment for fungal growth. The two species of fungi most frequently associated with field crops are *Aspergillus flavus* (Link ex Fries) and *A. parasiticus* (Speare). *A. parasiticus* is most frequently found in the soil or plant parts associated with the soil, but *A. flavus* may be found above or below ground on the corresponding plant parts. The genotype of hybrids resistant to European corn borer (*Ostrinia nubilalis* Hübner) did not affect the level of aflatoxin production. Planting date did not consistently affect aflatoxin production, but environments did. Georgia, Mississippi, Tennessee, and Louisiana always had more incidence and higher levels of aflatoxin than Missouri or Iowa. Non-aflatoxin producing isolates of *Aspergillus* spp. inoculated onto the silks of the maize plant did not reduce the aflatoxin contamination by crowding out aflatoxin producing isolates. A problem with research on aflatoxin contamination in preharvest maize is to obtain consistent results within an environment. Lack of a technique for fungal inoculation seems to be the problem. Insects, including the European corn borer, corn earworm (*Heliothis zea* Boddie, maize weevil (*Sitophilus zeamais* Motschulsky), and an erophysid mite (*Eriophyes tulipae* Keifer), have been used to vector spores and create an environment for fungal growth by infesting the tip of the maize ear with them after it had been inoculated with fungal spores. The maize weevil was the most effective vector. Husk tightness has been shown to be an important trait of maize which contributes to reduced aflatoxin contamination. The trait should be included in maize breeding programs in the southern U.S. Maize germplasm sources which are less susceptible than germplasm of most maize cultivars have been identified. Progress in improving these sources and getting them into acceptable breeding materials have been hampered by the lack of adequate screening techniques. We are presently evaluating a technique of exposing the seed to *A. flavus* and subsequently testing for germination.

Sap Beetles and Their Control

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Sap beetles are well adapted for carrying mycotoxin-producing fungi to crops such as corn. They are synchronized with the crop, are resistant to mycotoxins, can carry spores externally and internally, and are attracted by the odors produced by fungi that produce mycotoxins. They feed on crop residues from the preceding year contaminated with mycotoxin-producing fungi and then carry the inoculum to the developing ears of corn in the milk stage. They will enter the ears by any means available, and will take advantage of holes made by corn earworms or European corn borers. They will leave the corn when it begins to dry down, and so are rarely found on ears at harvest.

Although insecticides are registered for controlling sap beetles on corn, the number of applications required makes this approach economically undesirable. Pathogens, parasites, and predators of sap beetles are known, but their impact appears limited at this time. Fermenting baits have been used to reduce the numbers of these insects entering crops, such as figs or tomatoes, but are variable in effectiveness and, typically, must be replaced weekly. A small-scale trial we performed in 1989 indicated this method could be used in corn to reduce the incidence of mycotoxin-producing fungi (primarily *Fusarium*) in Illinois. In 1990, a totally artificial bait that was effective for at least 1 month was developed and used to reduce the incidence of these insects and of mycotoxin-producing fungi (primarily *Fusarium*) in three separate experiments involving 11 plots. Of four 1- to 2-acre plots of comparable conditions, ca. 1% of corn ears in two unprotected plots were found contaminated with *Aspergillus flavus*, while no *A. flavus* was found in two protected plots.

Corn varieties with good husk coverage can help reduce invasion of sap beetles. We found varieties with "open" vs. "cupped" leaf axles would accumulate less pollen, which the sap beetles feed on before moving into the ears. We found that some of the same corn chemicals that inhibit mycotoxin-producing fungi also inhibited feeding of sap beetles. Kernels from *Fusarium*-resistant varieties supplied by CIBA-GEIGY Seed in 1990 were also more resistant to sap beetles compared to susceptible varieties. Kernel tests performed in 1989, and ear tests performed in 1990 indicated an *A. flavus*-resistant variety (supplied by Gene Scott of Mississippi State University) was also more resistant to sap beetle feeding than a susceptible variety. This type of cross resistance should be highly useful in preventing mycotoxin contamination of corn.

CLIMATE AND WEATHER FACTORS AS THEY INFLUENCE AFLATOXIN CONTAMINATION IN CORN

Neil W. Widstrom

Aflatoxin contamination of the maize, Zea mays L., crop is a chronic problem in the southeastern U.S., but varies greatly from year to year in its severity. Our objectives were to study seasonal patterns of contamination for knife-wound inoculated ears as they related to several planting dates at Tifton, GA, throughout the growing season, and to relate the contamination to several environmental variables. Five plantings of B73 x Mo17 were made in 1982, six in 1983 and 1984, and ten were made in 1986 and 1987. Average planting dates varied from late February to late July. Eight to twelve 10-ear samples were assayed per planting date, after harvest at 60 d post-silking. Insect damage and the percentage of ears with visible Aspergillus flavus Link ex Fries were not consistently or strongly related to aflatoxin contamination, but maximum and minimum daily temperatures and net daily evaporation during 20-d periods following inoculation but prior to harvest were consistently related to the contamination process. Combined and average correlations for these characters were highly significant. Regression of aflatoxin amounts on planting date revealed a significant linear decrease in contamination from early to late plantings. Simple coefficients of determination of 0.75 or greater suggest a rather strong relationship when measured over a period of more than one year. Early plantings are believed to be at higher risk because the critical grain-filling period beginning 20 d after flowering falls during the time when seasonal maximum and minimum temperatures are highest and when net evaporation is also at its peak.

Field release of an atoxigenic strain of Aspergillus flavus

P. J. Cotty, USDA, ARS, Southern Regional Research Center, P.O. Box 19687, New Orleans, Louisiana 70179

Aflatoxin contamination of agricultural commodities might be reduced by qualitatively altering populations of Aspergillus flavus. Aflatoxins are not required for fungal infection of developing cotton bolls and corn ears. Therefore, naturally occurring atoxigenic strains might be useful in displacing toxigenic strains during infection of crops. This displacement should result in reduced aflatoxin contamination of the crops. In greenhouse experiments on cottonseed and field inoculation experiments on corn, atoxigenic strains have been very effective at reducing aflatoxin contamination. A test to evaluate the efficacy of an atoxigenic strain in cotton naturally infected by an endemic toxigenic A. flavus population was performed in the Yuma Valley in 1989. High levels of aflatoxins were encouraged in the plots by growing cotton each season with a winter fallow over a three year period and by permitting high levels of pink bollworm damage. In 1989, an atoxigenic biocontrol strain of Aspergillus flavus was released in these field plots early in the cotton season to permit the strain to compete for resources and participate in the increase in A. flavus propagules which occurs during the cotton season. Infection within the test plots was assessed by determining the incidence of locules with lint bearing bright-green-yellow-fluorescence. The incidence of the released atoxigenic strain in the harvested crop was determined by vegetative compatibility group analysis. The results indicate that population displacement is possible and that the atoxigenic strains might be useful tools for the management of aflatoxin contamination. Domestication of facultative plant pathogens such as Aspergillus flavus may provide the opportunity to address several problems associated with agriculture which we have not previously effectively managed. In order to use domesticated strains, we will have to develop a new technology to introduce and maintain the selected microflora. This technology will require incremental selection and improvement of strains in a manner partially analogous to plant cultivar development. Strain improvement will eliminate detrimental characters and increase useful traits. This strategy may permit management of aflatoxin contamination of both wounded and sound seed, both prior to and after harvest.

Biocompetitive Approach to Aflatoxin Prevention in Peanuts

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Studies on prevention of preharvest aflatoxin contamination of peanuts with non-toxigenic strains of Aspergillus parasiticus have been conducted at the National Peanut Research Laboratory (NPRL) since 1987.

The NPRL studies have utilized highly competitive non-aflatoxin-producing strains of A. parasiticus, which replace the wild toxic strains of A. parasiticus and A. flavus. This is achieved without a dramatic increase in fungal propagules above that which would normally be present in the soil. The major advantage with this strategy is that the non-toxic strains of A. parasiticus or A. flavus occupy the same ecological niche as the toxic strains. The use of other biocompetitive agents such as bacteria would also appear to be an attractive strategy; however, these become inactive under the extremely hot and dry conditions associated with preharvest aflatoxin contamination and thus do not occupy the same or a similar ecological niche. Therefore, they are not ideal biocompetitive agents for this application. The only possible impact these agents could provide would be production of inhibitory chemicals secreted into the soil during time when soil conditions are favorable for bacterial growth.

At NPRL we have a powerful experimental facility available that provides excellent control of soil moisture, soil temperature and soil microflora. The environmental control plot facility (ECPF) was utilized initially to elucidate environmental parameters responsible for preharvest aflatoxin contamination of peanuts. The facility now provides a valuable tool to develop and test preharvest prevention strategies, including the use of biocompetitive agents (BA).

A brief summary of studies at NPRL using non-aflatoxigenic strains of A. parasiticus follows. In 1987 a BA was added to one of the environmental control plots to test the concept. The study was conducted over a three-year period. No additional BA was added to the soil the two subsequent growing seasons (1988-1989). Each of the three years the peanuts were subjected to ideal conditions for preharvest aflatoxin contamination, harvested and subsequently analyzed for aflatoxin. Populations of both BA and wild toxigenic strains were monitored. The results of the aflatoxin analyses demonstrated a dramatic reduction in the level of aflatoxin contamination all three years when compared to peanuts from non-BA treated soil. The three-year study provided evidence that the concept is effective and justifies continued research to develop and refine this prevention strategy.

Studies conducted in CY 1990 included the evaluation of a new mutant of A. parasiticus in both the ECPF and several half acre plots in peanut fields. The objectives in the ECPF were to compare a new application technique using only fungal sclerotia at various rates (1, 5, 10 and 20 lb/acre). The field tests were designed to monitor the mutant fungus over several years, different soil types and crop rotation schemes. The fungus was applied at rates of 8-12 lb/acre of sclerotia with a gandy applicator. These studies have not yet been completed.

Proof of concept has been achieved and future studies will be designed to refine the technique for field application.

Biological control of Aspergillus flavus and Aspergillus parasiticus by Paecilomyces lilacinus.
D.M. Wilson and M.E. Will. University of Georgia, Tifton, GA
31793.

There were two objectives for this research in 1990. The
first was to determine if *P. lilacinus* could become a mycoparasite of
sclerotia and mycelia of *A. flavus* and *A. parasiticus* under laboratory
conditions. The second was to define the colonizing ability of
the *P. lilacinus* strains previously isolated from naturally colonized
sclerotia in Georgia and Illinois. Significant progress has been
made with the laboratory studies. The sclerotia buried in
'teaballs' in Illinois and Georgia were dug in November, 1990,
and these studies are just beginning.

Mycelial cultures of *A. flavus* and *A. parasiticus* were challenged
with *P. lilacinus, Trichoderma* species, and *Gliocladium* species using petri
dishes containing PDA or water agar and microscope slides thinly
coated with PDA or water agar. The *P. lilacinus* isolates generally
did not inhibit mycelial growth on PDA, and the mycelia appeared
normal. On water agar the *P. lilacinus* grew and sporulated while the
A. flavus and *A. parasiticus* only germinated. On PDA a few of the
Paecilomyces as well as the *Trichoderma* and *Gliocladium* isolates
inhibited *A. flavus* and *A. parasiticus* growth suggesting that some
diffusable antifungal metabolites were produced. More research
is planned using mycelial challenge studies of these potential
biological control fungi.

All *P. lilacinus, Trichoderma,* and *Gliocladium* isolates seemed to
invade, or at least colonize, *A. flavus* and *A. parasiticus* sclerotia when
sclerotia were inoculated using an aqueous spore suspension and
then placed at 25-30°C on moist sand with an a_w of .95 or above.
The sclerotia did not germinate while the other fungi sporulated
profusely on the surface of the sclerotia.

The field experiments in Georgia and Illinois, where
'teaballs' containing *A. flavus* or *A. parasiticus* sclerotia received
various *P. lilacinus* inoculation, chitin and/or cellulose amendment
treatments, were dug in November 1990. No evaluations are
presently available.

The results so far suggest that *P. lilacinus, Trichoderma* and
Gliocladium species may not be efficient mycelial mycoparasites,
but some isolates may produce diffusable inhibitory substances.
The *P. lilacinus* isolates apparently are able to grow and sporulate
with minimal nutrition (water agar). *P. lilacinus, Trichoderma* and
Gliocladium species all apparently colonize *A. flavus* and *A. parasiticus*
sclerotia and inhibit sporogenic sclerotial germination. These
preliminary experiments were initiated in May, 1990, and further
studies are planned.

<u>Title of the Project</u>: Management of aflatoxin contamination of cottonseeds using seed-borne endophytic bacteria.

<u>Investigators</u>: I.J. Misaghi and P.J. Cotty

Aflatoxin contamination of cottonseed is a chronic problem in Arizona that significantly reduces the market value of the crop. Such contamination mostly occurs in pink bollworm-damaged bolls following infection of the damaged tissues by *Aspergillus flavus* (1). No effective control measure has yet been developed for this important problem. Foliar application of non-systemic fungicides or biological control agents is ineffective because infection sites inside pink bollworm exit holes are inaccessible to foliar sprays. Therefore, alternative methods of managing the problem are needed. A study was initiated to explore the possibility of using endophytic bacteria to manage aflatoxin contamination of cottonseeds.

We recently have isolated several non-pathogenic isolates of endophytic bacteria, belonging to four genera, from internal portions of field-grown cotton cultivars in Arizona. The endophytic nature of isolates belonging to *Erwinia* and *Bacillus* groups has been confirmed by inoculating germinated cottonseeds with antibiotic-marked mutants of these isolates and re-isolating them from the interior of roots, stems, flowers, bolls, and seeds of mature plants generated from these seeds (2). Because of their ability to multiply within cotton tissue and become seed-borne, cotton endophytes appear to have the unique potential for interfering with *A. flavus* development, blocking aflatoxin production, or breaking down aflatoxin.

The efficient performance of biological control organisms depends on their ability to maintain effective population sizes on and around infection courts prior to the arrival of the pathogen. This criterion is met by a few of our endophytes that are induced to multiply at high rates in wounded boll tissue up to three fold greater than those present in intact tissue. It is likely that the wounds caused by the departure of pink bollworm from bolls will provide a favorable environment for the growth of these endophytes enabling them to interfere with the growth of the fungus and/or with aflatoxin production. We have, thus far, identified a few bacterial strains which are capable of inhibiting colonization of cottonseeds by *A. flavus*. The promising strains, particularly those which multiply at high rates in wounded tissue, will be tested in the greenhouse and in the field for their ability to reduce aflatoxin contamination of cottonseeds.

1.   Cotty, P.J. and L.S. Lee. 1989. Aflatoxin contamination of cottonseed; Comparison of pink bollworm damaged and undamaged bolls. Tropical Science 29:273-277.

2.   Misaghi, I.J. and Donndelinger, C.R. 1990. Endophytic bacteria in symptom-free cotton plants. Phytopathology 80:808-811.

GENETIC VARIATION IN THE RESISTANCE OF VARIOUS CULTIVARS OF
PISTACHIO, PECAN, ALMOND, AND WALNUT TO <u>ASPERGILLUS</u> <u>FLAVUS/
PARASITICUS</u> AND DETERMINATION OF ENVIRONMENTAL AND
ECOLOGICAL FACTORS NECESSARY FOR STABLE AND EFFICIENT
PREHARVEST AFLATOXIN SUPPRESSION FACILITATED BY THE USE
OF ATOXIGENIC STRAINS OF <u>A</u>. <u>FLAVUS/PARASITICUS</u>.

Noel **F. Sommer**, Dept. of Pomology, Univ. of CA, Davis, CA,
95616 and **Themis J. Michailides** Dept. of Plant Pathology, -
Univ. of CA, Berkeley/Kearney Agri. Center, Parlier, Ca 93648.

Co-Investigators: Gale McGranahan, Dan E. Parfitt, Dale
Kester and Tom Gradziel, tree nut genetics.

Cooperators: R. J. Fortlage and D. P. Morgan, Staff Res. Assoc.

Potential biocontrol agents that have demonstrated
suppression of <u>Aspergillus flavus</u> in Petri dishes have
been collected for further studies. We are continuing
collecting, using an Andersen microbial sampler, in search
of greater suppressiveness.

Studies of infection courts for <u>A</u>. <u>flavus</u> in nuts
have shown that in pistachios, most of the aflatoxin
contaminated nuts has resulted from early splitting or navel
orange worm activity. Navel orange worms preferentially
attack early split nuts. Studies by Dr. Dan Parfitt
indicate considerable cultivar differences in pistachios
in the amount of early splitting but data for more than one
year will be required before conclusions can be drawn.

Early splitting in pistachios is a defect in which the
shell and hull split, exposing the kernel. Normally, the
shell splits after the hull has loosened. In the latter
case, the kernel is still enclosed within the hull.

Studies on the effect of the seed coats on resitance
to infection by <u>Aspergillus flavus</u> have been done with
pistachios and almonds. The seed coats of both species
have demonstrated considerable resistance to infection when
conidial suspensions of <u>A</u>. <u>flavus</u> were sprayed on sound
nuts. Under the same test conditions, broken or scratched
nuts were invariably infected and developed abundant
sporulation.

Dr. Tom Gradziel has suggested that resistance to <u>A</u>.
<u>flavus</u> infection by the seed coat might be an important
characteristic that might be modified by breeding.

Studies now underway are designed to determine: are
walnut and pecan seed coats similarly resistant to <u>A</u>.
<u>flavus</u>? Is the resistance physical or chemical?
Are there important differences between cultivars in the
resistance of the seed coat?

Safe and Acceptable Biocompetitive Approaches to Aflatoxin Elimination

Chair: J. L. Richard

Panel: P. Cotty, R. Cole, D. Wilson, I. Misaghi, and R. Fortlage

The development of biocompetitive approaches to eliminate or control aflatoxin is obviously one reasonable approach and we have heard some excellent presentations in this regard.

Within the U.S.D.A. and the Agricultural Research Service, technology transfer is an important facet of our research which includes the overall objective of increased utilization of farm commodities. Therefore, we as scientists cannot rest on the academic aspects of our research but must be prepared to follow our research effort to the point of solving problems to enable the technology to be transferred to industry.

Among the problems involved with biocompetitive agents is that the health and safety aspects of introducing a biocompetitive agent into the environment must be recognized. These agents can be pathogenic to animals, including humans, insects (some of which may be desirable), and perhaps other plants in the vicinity of the treatment crop. Will these agents occupy an ecological niche that is undesirable?

While most of the panel members recognized the necessity of eventually testing their respective organisms for pathogenicity in animals, and other facets mentioned above, they felt that their major effort should be directed to control of aflatoxin in their respective field crop, ie. peanuts, cotton, corn, and tree nuts. Although some panel members suggested that pathogenicity and other testing should come at a later time and may perhaps be the responsibility of the company or industry to which the technology has been transferred, it was felt that scientists have a major responsibility in this area. Agents such as Aspergillus parasiticus and certain bacteria that have no known pathogenicity for animals would be agents more likely to receive approval from regulatory agencies. It was noted during the discussion that A. flavus is the prodominant cause of aspergillosis in human patients particularly in large cities, therefore there should be concern when this organism is used as a biocontrol agent.

The use of microbial agents in large quantity for biocontrol requires that the investigator exercise care and sound judgement in applying the agent to field crops, to recognize that the organism may occupy niches other than the one in which he/she is interested, and to cooperate with those who could provide answers to questions regarding the pathogenicity or potential dangers involved with their respective agent. The entire aflatoxin problem is a multidisciplinary problem and answers to the safe and acceptable use of biocompetitive agents will require a multidisciplinary approach.

Bioregulatory control of aflatoxin contamination by elucidation of its biosynthetic process.

T. E. Cleveland and D. Bhatnagar, USDA, ARS, Southern Regional Research Center, P.O. Box 19687, New Orleans, Louisiana 70179

Perhaps the one feature all crops contaminated with aflatoxin have in common is that the toxin is synthesized in the seed by a complex biosynthetic pathway under currently unknown regulatory controls. An understanding of the regulation of the aflatoxin biosynthetic pathway could lead to methods to interrupt the process leading to toxin formation and provide "generic" methods for controlling aflatoxin contamination in all crops.

Many of the critical chemical and biochemical factors controlling aflatoxin formation by the fungus, Aspergillus parasiticus, have been elucidated (Figure). Several of the chemical intermediates in the aflatoxin pathway have been identified. Identification of aflatoxin pathway intermediates allowed these compounds to be used as enzyme substrates to detect aflatoxin pathway enzyme activities in sub-cellular fractions of fungal mycelia. Several critical pathway enzymes, including two methyltransferases, a reductase and an oxidoreductase have been identified, purified and characterized. Oligonucleotide and antiserum probes, synthesized based on the purified and characterized enzymes, are being utilized to study the molecular regulation of key pathway catalysts and to identify the genes coding for these aflatoxin pathway enzymes.

Cloning of aflatoxin pathway genes, along with the antibodies, will provide molecular probes for future applications in biotechnology involving: (1) identification of the aflatoxigenic potential of contaminating atoxigenic fungi before they produce the toxins and render the commodity unfit for consumption; (2) investigation of the regulatory factors (from the fungus and/or the host plant) controlling the onset of aflatoxin biosynthesis; (3) precision inactivation of specific steps/genes in aflatoxin biosynthesis will result in the production of specific aflatoxin non-producing fungi. Stable aflatoxin non-producing strains thus produced in the laboratory will be tested for effects of introduced mutations on their "fitness" in diverse environments and for their potential as biocompetitive agents.

FIGURE

Scheme of chemical intermediates and enzymes involved in aflatoxin biosynthesis:

NOR, Norsolorinic acid; AVN, averantin; AVNN, averufanin; AVF, averufin; VHA,

versicolorin hemiacetal acetate; VAL, versiconal; Ver A, versicolorin A; Ver C,

versicolorin C; ST, sterigmatocystin; DHST, dihydrosterigmatocystin; OMST, O-

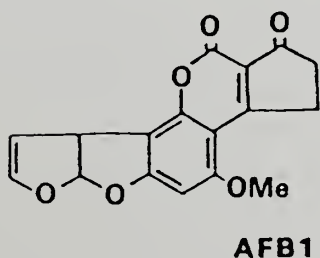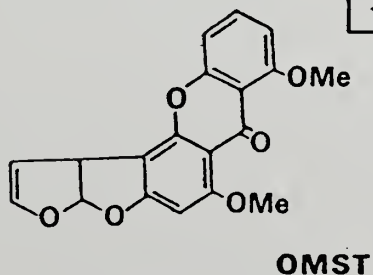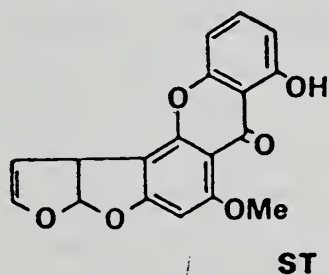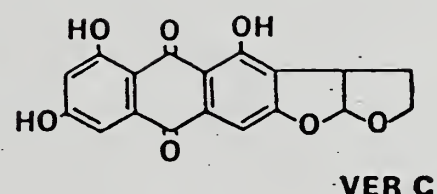methylsterigmatocystin; DHOMST, dihydro-O-methylsterigmatocystin.

POLYKETIDE

NOR

REDUCTASE

AVN

AVNN

AVF

VHA

ESTERASE

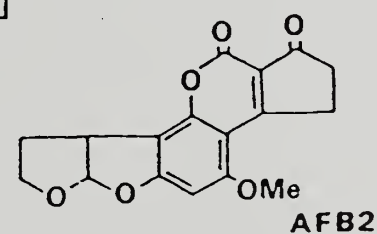VER A   VAL   VER C

ST   DHST
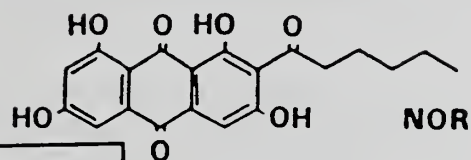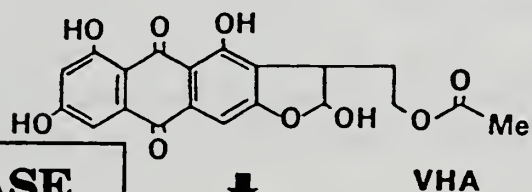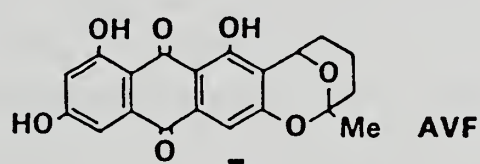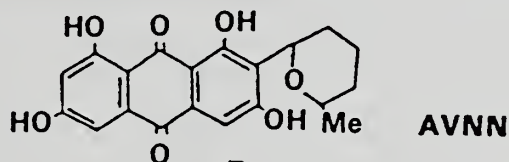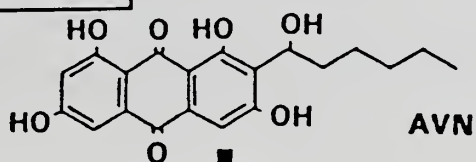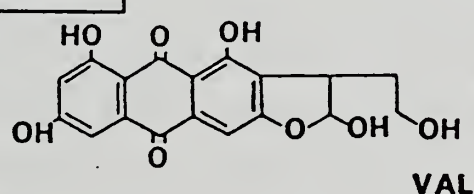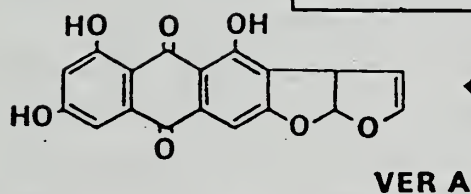
METHYLTRANSFERASE

OMST   DHOMST

OXIDOREDUCTASE

AFB1   AFB2
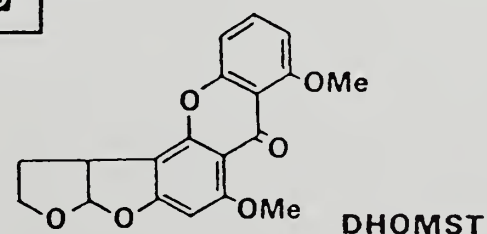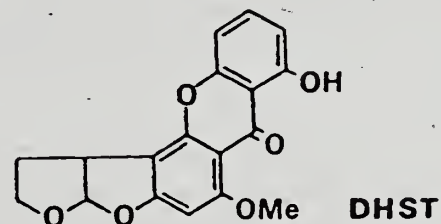
REPORT TO THE AFLATOXIN TECHNICAL ADVISORY GROUP

Gary A. Payne
North Carolina State University
November 1, 1990

Characterization of a Gene for Aflatoxin Biosynthesis

The goal of my research program is to isolate genes in the aflatoxin biosynthetic pathway and study their regulation. Although aflatoxin biosynthesis has been studied extensively and many intermediates in the pathway have been identified, regulation of the pathway is poorly understood. Studies on aflatoxin regulation have been arduous because pathway enzymes have been difficult to isolate and characterize. Rather than attempt to study pathway regulation through enzyme isolation and characterization, I have chosen to elucidate the regulation of aflatoxin biosynthesis by directly isolating genes in the pathway.

We are using a genetic transformation system we developed for A. flavus to identify genomic clones that contain the genes for aflatoxin biosynthesis. Using this strategy we identified one genomic clone (B9) that when transformed into the blocked mutant 650-33 restored the ability of this strain to produce aflatoxin. Aflatoxin production by individual transformants ranged from 5 to 1616 ug aflatoxin B1 per ug dry weight, and depended on the number of copies of B9 incorporated into the genome of the transformants. Transformants containing from 1-3 copies of the B9 gene produced more aflatoxin than strains with 4-6 copies of the gene.

One transformant with a single copy of B9 was chosen for further study. The onset and profile of aflatoxin production was similar for this transformant and a wild type strain. Aflatoxin was detected in cultures 8 hr after transfer in inducing medium and rapidly accumulated from 16-32 hr before showing a slight decline. To determine if messenger RNA specific for gene B9 was expressed during aflatoxin biosynthesis, we isolated total RNA and hybridized it to a 4.1 kb EcoRI fragment of B9. mRNA that hybridized specifically to the 4.1 EcoRI fragment could be detected at 8 hr and its concentration peaked at 16-24 hr. No hybridizing mRNA was seen beyond 32 hr or at any time from cultures grown in non-inducing medium.

We are in the process of sequencing this gene for aflatoxin biosynthesis. The sequence will provide information on the protein product of the gene and will help define the promoter region of the gene. Once the promoter region of the gene is characterized we will begin to study the regulation of the gene and examine factors that influence its regulation.

Techniques for Identification of Resistance to Preharvest Aflatoxin Contamination in Peanut C. C. Holbrook, USDA/ARS, Tifton, GA; D. M. Wilson, W. F. Anderson, UGA, Tifton, GA; M. E. Matheron, UAZ, Yuma, AZ; M. E. Wills, UGA, Tifton, GA; A. J. Norden, UF, Gainesville, FL; and R. W. Beaver, UGA, Tifton, GA.

There two objectives for this research in FY 1990. The first was to develop an effective and efficient technique for identifying resistance to preharvest aflatoxin contamination. The second was to select a core collection which is representative of the entire peanut germplasm collection and to increase seed of this collection for screening in FY 1991. Significant progress has been made on both of these objectives.

Approximately two acres of peanuts were grown in Yuma, AZ to evaluate this location for its appropriateness as a screening site. One experiment using Florunner and two planting dates was designed to determine if consistent results can be obtained at Yuma. This study was also designed to provide information on the best resource allocation (plot size and replication number) for future screening studies. An additional study using four genotypes (Florunner, Pronto, Tifton-8 and J-11) and two planting dates was conducted to determine if moderate levels of genetic differences for preharvest aflatoxin contamination can be determined at Yuma. A study was also conducted to determine if shade cloth can be used to improve the screening technique by reducing soil temperature. Quantitative aflatoxin measurements will be taken on harvested kernels.

Studies grown under rain protected shelters and in the greenhouse at Tifton, GA were conducted to determine the best time and rate for inoculation. These studies were also designed to examine the use of corn as compared to water as a carrier for the inoculum. A greenhouse study using four genotypes (Florunner, Pronto, Tifton-8 and J-11) and two inoculation treatments was also conducted to determine if moderate levels of genetic differences can be detected under greenhouse conditions. Quantitative aflatoxin measurements will be taken on harvested kernels. Additional greenhouse and laboratory studies are underway to define the mechanism of resistance in Tifton-8 and J-11.

The U.S. germplasm collection for peanut consists of over 7,500 different genotypes selected from throughout the world. All the available data on these genotypes was obtained from Dr. Roy Pittman, the curator for the collection. Multivariate statistical analysis was used on this data set to cluster the genotypes into genetically similar groups. Random sampling was then used to select approximately ten percent from each group. The resulting 831 genotypes form the Core Collection for peanut. Data for this collection indicates that most of the genetic variability from the entire collection has been maintained in the core collection. Seed for genotypes in the Core Collection were increased to screening for resistance to preharvest aflatoxin contamination in 1991.

Breeding for Resistance to Apsergillus flavus

Gene E. Scott, USDA-ARS, Mississippi State, Mississippi

The objective of our breeding program is to develop corn genotypes which resist kernel infection by A. flavus and subsequent aflatoxin production. When this program was initiated, there were no known sources of resistance and no known inoculation techniques. In Mississippi, we have developed the pinbar inoculation technique and a side-needle inoculation technique where a tree-marking gun with a hyperdermic needle is used to put the conidial suspension between the husks and kernels with minimal kernel damage.

When we inoculated two resistant x resistant hybrids and two susceptible x susceptible hybrids, the susceptible hybrids had more than twice as many kernels infected and much higher aflatoxin contamination than the resistant genotypes. In later tests, we tested five inbreds designated resistant and five susceptible. Less than 10% kernel infection was present in the resistant inbreds compared to 30% in the susceptible inbreds. When we took five resistant lines and crossed them in all possible combinations and five susceptible lines and crossed them in all possible combinations, the resistant crosses had considerably less kernel infection by A. flavus than did the susceptible genotypes.

In a separate study involving two resistant and two susceptible genotypes planted at three planting dates in one year and either inoculated with A. flavus, A. parasiticus, or not inoculated, genotypes resistant to A. flavus were also resistant to A. parasiticus.

When dealing with a kernel invading fungus, the seed is one generation past that of the plant producing the seed. If an F_1 generation is grown in the field, the F_2 generation is evaluated except for the pericarp. When an inbred is selfed, the seed produced on that inbred is inbred. But if that inbred is crossed, F_1 seed is produced on that inbred. If the F_1 is silked, an F_2 population is produced. Thus, when evaluating self pollenated hybrids, the F_2 generation has actually been evaluated. In experimental plots that are open pollenated, the female portion of the seed is seed of an F_2 and the male is just whatever pollen is there. And if the F_1 is a resistant x susceptible cross, then actually a segregating F_2 is tested. So maybe a lot of the times when looking at hybrids, one of the parents may have had some resistance, the other susceptible and we get a lot of variation which we would expect when looking at an F_2. Therefore, we weren't able to detect the resistance.

If we go ahead and make reciprocal F_1's and reciprocal F_2's, and reciprocal backcrosses, and then evaluate all of this material, we can determine whether the site of action of the genes for resistance is in the pericarp, cytoplasm, endosperm or embryo. We now have inoculation techniques that can separate resistant from susceptible genotypes and we have identified some sources of resistance, and understand how ears and kernels are infected so that breeding for resistance to kernel infection in corn should now be more rapid and effective.

10-31-90

A new beginning in identifying maize genotypes for resistance to
Aspergillus flavus.

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The effort to identify maize genotypes resistant to A. flavus infection
and aflatoxin contamination has been disappointing because of our
inability to consistently detect significant differences among genotypes
being tested. In 1989 we began cooperative research with CIBA-GEIGY Seed
Division, Bloomington, IL. Our objectives are (1) to develop better
procedures for evaluating genetic resistance to A. flavus infection and
aflatoxin; (2) to contrast patterns of resistance/susceptibility to A.
flavus among inbred and hybrid genotypes with known reactions to specific
ear and kernel rotting fungi; (3) to construct a maize hybrid that
combines relevant morphological and physiological characters that provide
additive polygenic resistance to aflatoxin contamination.

A bright greenish yellow fluoresence (BGYF) appears in some kernels after
A. flavus becomes established in the germ and produces kojic acid. Simply
by counting the number of BGYF kernels surrounding individual
wound-inoculation sites we were able to pair several maize hybrids that
were planted in 1989 as part of a blind screen. There were consistent
differences in the numbers of BGYF kernels produced on ears of selected
inbreds and hybrids.

Evidence obtained from pathogenicity trials in controlled environments and
from studies on the physiology of kernel filling, shows that temperature
stress during kernel filling produces random breaks in the internal seed
coat (testa) giving A. flavus access to the germ. No evidence was found
that A. flavus hyphae can enter kernels through the black layer, point of
silk attachment, or any region of an intact seed coat. It is well
documented that increased losses in maize from insect damage can be
attributed to new early maturing hybrids with loose, open husks and quick
dry-down attributes that have recently been introduced in the southeast.
A hypothesis is offered that these hybrids, initially developed for the
midwest Corn Belt, are also poorly adapted to temperature stress during
kernel filling. This could be a simple explanation for the increased
magnitude of aflatoxin outbreaks in the southern United States.

Distribution of Aflatoxin Contamination Among Single Kernels from Individual Plants and Among Single Plants

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Aflatoxin contamination of peanuts is a highly variable phenomenon evidenced by the fact that in a contaminated lot of peanuts, very few of the kernels are actually contaminated. Studies were conducted over three crop years to determine possible sources of this variability at the field level to gain further insight into the basic mechanisms of aflatoxin contamination and determine possible bases for genetic resistance and/or susceptibility.

The first year, 1340 individual kernels were analyzed from 44 Florunner plants subjected to drought conditions optimized for aflatoxin contamination. Results showed that only 163 (12.2%) kernels had detectable levels of aflatoxin and only 47 (3.5%) contained greater than 20 ppb of aflatoxin. There appeared to be an association of aflatoxin contamination with a few individual plants as about 90% of contaminated kernels came from only 9% of the plants. In the case of one particular plant, all kernels but one contained aflatoxin with about 60% of the kernels contaminated above 10 ppb. There was also an association between aflatoxin contamination and the condition of plants under drought stress. The most severely stressed plants were less likely to have aflatoxin-contaminated kernels than less stressed plants. A possible reason for this is that when a peanut plant dies relatively quickly, the water activity of the peanuts on that plant decreases much more rapidly; thus, those peanuts are not in a susceptible water activity range long enough for A. flavus growth and aflatoxin formation to occur. On the other hand, a more drought tolerant plant maintains higher peanut kernel water activities longer, thus increasing the time that peanuts are in a susceptible water activity range favorable for A. flavus growth and aflatoxin formation. Finally, this study corroborated earlier results that showed that most of the aflatoxin contamination was associated with the more immature peanuts.

In the second year, all peanuts from individual plants of the Florunner cultivar and a white-seeded cultivar were analyzed after subjection to late-season drought stress. Results showed that for 235 Florunner plants, 98% of the total aflatoxin came from 5% of the plants. For the 252 white-seeded cultivar plants, 99% of the aflatoxin came from 4% of the plants. In the third year, each of the three individual Florunner sister lines were planted separately along with the susceptible Makula Red cultivar. Results showed that >99.5% of the total aflatoxin contamination for each was associated with 17-19% of the plants.

Results from these studies have shown that although all peanut plants were exposed to ideal conditions for aflatoxin contamination, a very small percentage of plants actually had contaminated kernels, and very few kernels were contaminated. This seems to indicate that peanuts generally are quite resistant to aflatoxin contamination and the chances for a particular kernel to become contaminated are quite low. The susceptibility of a peanut kernel/plant to A. flavus/parasiticus seems to be a matter of being in the right place at the right time. The fungus must be present and viable in the peanut when opportunities for growth and aflatoxin formation are present. If peanuts are naturally resistant and contamination results more from timing and opportunity than from genetic susceptibility, it seems that the likelihood of finding a genotype that possesses complete and absolute resistance is remote.

Identification of Molecular Markers Associated with Genes for Preharvest Resistance in Corn to Aspergillus flavus and aflatoxin production.

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The major objective of our research is to identify higher levels of resistance to Aspergillus flavus and aflatoxin production in maize germplasm adapted to the Corn Belt than is presently available. The summer of 1990 we crossed over 1200 inbreds onto the inbreds Mo17 and B73. Mo17 and B73 represent the genetic basis of a large percentage of current Corn Belt hybrids. Mo17 and B73 do not express high levels of resistance to Aspergillus flavus and aflatoxin production. In 1991, we intend to plant the new F1 seed and to inoculate the resultant plants with Aspergillus flavus isolates obtained from Dr. Don Wicklow, USDA-ARS, NRRL, Peoria, IL.

The maize inbreds that we crossed this past summer have very diverse genetic backgrounds. These lines include releases from Agricultural Experiment Stations, many of which were selfed out of old open pollinated varieties. Also included are inbreds from Canada, Europe, India, Mexico, Northern China, and South Africa. Some of the various sources of identified resistance in the Southern United States were included.

We evaluated different methods of inoculation with Aspergillus flavus isolates in the summer of 1990. We are currently developing new prototypes of inoculation mechanisms that will be suitable for the large scale of inoculations that will be performed in 1991.

The longer term objective of the project will be to identify molecular markers that are linked to genes for resistance to Aspergillus flavus and aflatoxin production in maize. This will require identifying a good source of resistance and then performing the necessary genetic and molecular genetic experiments and analyses. The advantage of identifying molecular markers associated with resistance to Aspergillus flavus is that breeders could potentially screen for resistance in years when environmental conditions are not suitable for accurate screening. Furthermore, molecular markers may facilitate the transfer of genes for resistance from nonadapted germplasm into elite commercial hybrids.

BREEDING FOR AFLATOXIN RESISTANCE IN PEANUT

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Breeding for lowered aflatoxin levels in peanut can target selection in the field and/or during seed storage. Three mechanisms of resistance have been reported to reduce aflatoxin contamination, including preharvest infection resistance (PIR), dry seed resistance (DSR), and aflatoxin production resistance (APR). To evaluate the genetic effects of the three types of resistance, two cultivars (NC 7 and Gajah) were each crossed with two reportedly resistant genotypes (J11 and U 4-7-5). *Aspergillus parasiticus* was applied to rehydrated seeds (DSR), seeds with the testa removed (APR), and to greenhouse-grown plants subjected to drought stress (PIR). Estimates of gene action were then calculated using the generated means analysis. For DSR, additive genetic effects were most important and selection should be effective using early generation hybrids. Additive x dominant epistatic effects were significant for APR which means that selection should be carried out in late generation. Further, selection of favorable genotypes will probably be difficult. Variable effects were observed for PIR and methodology needs to be improved for future evaluations in the greenhouse.

Heritability estimates were obtained using families of several crosses in the $F_{4,5}$ generation. DSR had a significantly high value and selection should be effective for this trait. Aflatoxin production and PIR had low heritabilities and selection will be more difficult. Correlations between the three mechanisms were nonsignificant, indicating independent genetic control. Families with multiple resistances were observed during the study, which is promising for making future progress toward decreasing aflatoxin levels.

Because levels of resistance in *A. hypogaea* are not high, 68 accessions of *Arachis* species, two amphidiploids and four cultivated checks, were tested for DSR. All accessions had at least 30% of their seeds infected after inoculation with *A. parasiticus*. Several accessions had relatively low levels of *Aspergillus* infection on a per-seed basis, but none were significantly less than the *A. hypogaea* line J11. The results indicated that, among the wild species accessions tested, usable levels of dry seed resistance are not available.

Aspergillus flavus and Fusarium moniliforme infection in kernels of field
inoculated maize ears

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When transversely cut kernels from ears needle-inoculated in the field with
Aspergillus flavus were assayed, significantly more pedicel than apical
portions were infected with Fusarium moniliforme. In contrast, infection by
A. flavus was detected more frequently in apical sections. In addition, A.
flavus was isolated more from middle portions of inoculated kernels than from
the pedicel ends. The recovery of A. flavus and F. moniliforme from apical
and pedicel portions of kernels was not affected by genotype or inoculation
method (needle or pinbar).

Aspergillus flavus was recovered from a high percentage (83%) of cobs in both
inoculated and uninoculated ears of all maize genotypes. Aspergillus flavus
was recovered from a higher percentage of placental and sclerenchymatous
tissue than from pith segments. Fusarium moniliforme was isolated from cobs
less frequently than A. flavus but appeared to be more uniformly distributed
within cob tissues. The correlation (r values) of kernel infection by A.
flavus with colonization of placenta and sclerenchyma in the cob was
statistically significant, but r values were quite low. The relatively low
level of pedicel infection of kernels by A. flavus (7%) compared to combined
infection of other kernel segments (45%) strongly indicates that A. flavus
infects maize kernels through the pericarp.

Maize hybrids that were resistant to kernel infection by A. flavus following
inoculation were also resistant to kernel infection by A. parasiticus. There
was a significantly lower incidence of infected kernels from resistant
cultivars over all planting dates. Aspergillus flavus appears to have a
greater capacity for survival in maize cob debris than does A. parasiticus.
Conidia of A. flavus were the predominant aspergillus spores sampled in maize
fields when ears were developing indicating that A. flavus may present greater
potential for serving as inoculum for natural infection of maize kernels than
A. parasiticus.

SOME RESULTS FOR SEPARATION OF AFLATOXIN AND DEOXYNIVALENOL CONTAMINATED KERNELS.

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Abstract

In recent years, grain infection and contamination by myco- and other toxins have become serious problems having a very large health and economic impact. As an example, aflatoxin contamination has been a recurring problem in the peanut and corn industry since its discovery in 1960. Government regulations in the USA and Europe tend to limit and reduce the aflatoxin level allowed.

Different methods have been used by researchers to decrease the level of mycotoxin contamination of grain products. These methods included agronomical, ecological and biotechnological practices, and various separation systems. The presented work reflects attempts to decrease aflatoxin and deoxynivalenol levels in peanuts, corn and wheat using a dry density separation method with high density accuracy of 1-2 percent, developed by Sorting Technology, Inc., Pocatello, Idaho.

Many researchers have observed the relationship between the level of aflatoxin contamination and a product's specific gravity. This relationship includes such products as peanuts, corn, soybean seeds, interalia. However, the only practical existing method for sorting contaminated kernels from sound ones has been separation in different kinds of liquids, which is difficult for commercial application. Currently, industry does not have commercial dry density separator. A gravity table often is referred to as a density separation, however, research has shown that this may not be true because the drag coefficient may predominantly influence the separation. The density accuracy of a gravity table is 13 to 15 percent (according to the literature and tests carried out by Golden Peanut Company). A gravity table is not suitable for sorting aflatoxin contaminated kernels from good kernels due to its unsatisfactory accuracy level of density separation.

The tests carried out with peanut, corn, and wheat kernels on both a laboratory batch type machine and the continuous process machine demonstrated very attractive results. They were successful in concentrating the aflatoxin contaminated kernels into a rejected product composed of 1 to 3 percent of the entire product. The recovered product, which was 97 to 99 percent of the entire product, was practically free from aflatoxin. Parallel results were obtained for deoxynivalenol contaminated products.

The aflatoxin detection method included extraction and VICAM Immunoaffinity Column Cleanup with consecutive quantitation using a calibrated fluorometer. The deoxynivalenol (DON) detection method consisted of extraction and cleanup on an RP-C18 column. A UV detector set at 220nm was used to consecutively compare the DON peak height to that of a standard DON reading.

RESISTANCE TO AFLATOXIN CONTAMINATION IN Lfy CORN

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Preharvest aflatoxin contamination of corn (Zea mays L.) by Aspergillus flavus is a chronic problem in the southeastern USA. Aflatoxin-contminated corn ingested by livestock leads to poor health and/or death. There is also an increasing concern about the effects of aflatoxin on human health. The most effective way to control aflatoxin contamination in corn is through host-plant resistance. We conducted two field experiments to 1) study the genetics of resistance to aflatoxin production in corn with the Lfy gene and 2) compare aflatoxin production by A. flavus vs. A. parasiticus using silk inoculation.

In the first study, progeny of 21 single crosses from a diallel mating of seven synthetics (A632, A619, B73, Mol7, HY, Wf9, 914) containing the Lfy gene were grown at the Ben Hur Research Farm in Baton Rouge, LA. Twenty-one days after mid-silk, ears were inoculated by dipping a knife into a suspension of 20 X 10^6 spores/ml of A. parasiticus and then cutting through the husk and injuring one kernel row. Ears were harvested 5 weeks after inoculation and dried for 3 days at 65°C. Two kernel rows adjacent to the injured row on each ear were hand-shelled, bulked for each plot, and assayed for aflatoxins.

General combining ability (GCA) mean squares were greater than specific combining ability (SCA) mean squares, suggesting that additive genetic effects control aflatoxin production more than non-additive genetic effects in these genotypes. Negative estimates of GCA effects for genotypes A632, B73, and Wf9 indicated that, on average, crosses with these genotypes would have lower aflatoxin contamination than crosses among other genotypes. Since GCA was more important than SCA, a recurrent selection program may be effective in increasing aflatoxin resistance in these genotypes. Further testing is being done.

In a second field study, conducted at two locations in Baton Rouge, seven genotypes containing the Lfy gene were inoculated with either A. flavus or A. parasiticus to determine the difference, if any, in aflatoxin production between the two species. Silk inoculation was done by atomizing a 2 ml solution of 20 X 10^6 spores/ml of either fungal species on separate plots at 2 and 3 weeks after mid-silk. Plots were harvested 5 weeks after inoculation. Ears were dried at 65 C for 3 days. Ears were machine shelled, bulked, and assayed for aflatoxin production.

Results indicated that A. flavus produced a significantly higher amount of aflatoxin than did A. parasiticus. Evidently, little or no infection by A. parasiticus occurred via silk inoculation. Aflatoxin production was consistent between the 2 locations. This inoculation technique did not produce high enough aflatoxin concentration to differentiate among genotypes.

Inhibition of some mycotoxigenic fungi by Iturin A, a peptidolipid
produced by Bacillus subtilis.

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The current concern about potential environmental damage by commercial
fungicides has led to an increasing demand for new control methods.
Bacillus subtilis produces peptidolipid compounds of the iturin group which
have been shown to have antifungal properties, but not all fungal species
are sensitive to these compounds. In this study, the activity of iturin A,
produced by B. subtilis strain B-3, was tested. Paper disks impregnated
with various concentrations of iturin A were placed on agar plates seeded
with conidia of toxigenic species of Fusarium, Penicillium or Aspergillus.
Most isolates were inhibited at iturin A concentrations as low as 4 ug/disk.
Penicillium italicum, P. viridicatum and A. ochraceus were most strongly
inhibited by the iturin, whereas P. citrinum, A. flavus, A. parasiticus, and
F. moniliforme were least sensitive to iturin A.

Effects of cotton ovule stress metabolites on growth of <u>Aspergillus flavus</u>
and aflatoxin production.

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Developing cottonseed is a target for the toxigenic fungus <u>Aspergillus</u>
<u>flavus</u>. A number of parameters associated with fungal production of the
potent carcinogen aflatoxin (AFB-1) are poorly understood. A whole family
of antifungal sesquiterpenoid metabolites has been isolated from cotton leaf
and pith tissues. Developing cottonseed tissue also has the capacity to
produce antifungal stress metabolites in response to <u>A</u>. <u>flavus</u> infection.
An investigation was undertaken to determine the effects of cotton ovule-
derived stress metabolites on <u>A</u>. <u>flavus</u> growth and subsequent production of
AFB-1. A crude stress metabolite mixture was obtained by extraction of
<u>A</u>. <u>flavus</u>-inoculated cotton ovule cultures with acidified ethyl acetate.
Extracts derived from either non-inoculated cotton ovule tissue or cotton
ovule tissue challenged with <u>A</u>. <u>flavus</u> inhibited AFB-1 synthesis in fungal
cultures of <u>A</u>. <u>flavus</u>. Extracts derived from fungal-challenged ovule
tissue, when added to fungal cultures in concentrations of 50 µg per mL of
medium, inhibited AFB-1 synthesis by as much as 93%. The ED_{50} for this type
of extract was 18 µg per mL of medium (P<0.01; r^2=0.46). Similar experi-
ments with a non-inoculated ovule extract (50 µg/mL medium) produced AFB-1
inhibition levels of up to 77%. The ED_{50} for non-inoculated extracts was
35 µg per mL of medium (P<0.01; r^2=0.66). These extracts did not inhibit
the growth of <u>A</u>. <u>flavus</u> in culture. The data suggests that both inoculated
and non-inoculated cotton ovule tissues contain components which are
inhibitory to fungal aflatoxin production, but not to fungal growth.

Antifungal polypeptides and enzymes in corn implicated in resistance to
Aspergillus _flavus_ infection.

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The nature of fungal growth inhibition (_Aspergillus_ _flavus_) by buffered
saline-soluble kernel proteins in highly susceptible (Huffman) and highly
resistant (Yellow Creole) open-pollinated varieties of maize was studied.
For both varieties, the isocratic protein fractions from preparative
anion-exchange chromatography showed sporadic fungistatic effects in solid
agar media. In both solid and liquid media, chromatographic fractions
immediately following isocratic elution showed fungal growth inhibition only
in the resistant variety. The two varieties were also assayed for chitinase
activity. Results showed higher activity in the germs than in the endo-
sperms with disparity between the two varieties. The highest activity per
quantity of protein was observed in the germ of the Huffman variety and in
salt fractions precipitated with 20% ammonium sulfate. From gel permeation
chromatography, major chitinase activity occurred in the fractions with
molecular size ranging from 14 to 25 kilodaltons. Isoelectric focusing on
acrylamide gels showed major chitinase activity in protein zones at pH 3-4.
These data provide evidence that the catalytic components exist in several
isozymic forms that are acidic and highly associated in the native state.
Their possible role in protecting corn from _A_. _flavus_ infection and
aflatoxin contamination, and their specific interactions with cell walls of
fungal spores, however, remain to be determined in future studies.

A profile of defense expression in the cotton plant upon challenge from
toxigenic strains of <u>Aspergillus</u> <u>flavus</u>.

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The following are representative of natural defense factors present in the
cotton plant; methods used to enhance their amounts should increase the
defense(s) of the cotton plant against <u>Aspergillus</u> <u>flavus</u> infection and
ultimately against aflatoxin contamination.   Cotton leaves or cotton bolls
challenged with cell-free mycelial extracts (elicitor) of toxigenic strains
of <u>A</u>. <u>flavus</u> produce five auto-fluorescent phytoalexins: lacinilene C,
lacinilene C 7-methyl ether, scopoletin, 2-hydroxy-7-methoxycadalene, and
2,7-dihydroxy cadalene.   Wounding the cotton leaf results in fungistatic
accumulations of ferulic acid; wounding and treatment of the leaf with
elicitor results in elevated levels of the coumarin phytoalexin, scopoletin.
"Gaseous phytoalexins," fungicidally potent C_6-C_9 alkenals derived from
leaf component linoleic and linolenic fatty acids, are also liberated from
the microbial damaged leaves.   Volatiles originating from <u>A</u>. <u>flavus</u>-infected
cotton leaves trigger significant defensive chemical changes in undamaged
cotton leaves sharing the same gaseous enclosure.   The bioactivity of
selected, individual purified cotton leaf head space volatiles in cultures
of <u>A</u>. <u>flavus</u> were demonstrated.